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ORIGINAL ARTICLE

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Loss of β1-integrin-deficient cells during the development of endoderm-derived epithelia

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Abstract β1-Integrins (β1) represent cell surface receptors which mediate cell–matrix and cell–cell interactions. Fässler and Meyer described chimeric mice containing transgenic cells that express the *LacZ* gene instead of the *β1* gene. They observed β1-negative cells in all germ layers at embryonic day E 8.5. Later in development, using a glucose phosphate isomerase assay of homogenized tissue samples, high levels of transgenic cells were found in skeletal muscle and gut, low levels in lung, heart, and kidney and none in the liver and spleen (Fässler and Meyer 1995). In order to study which cell types require β1 during development of the primitive gut including its derivatives, chimeric fetuses containing 15 to 25% transgenic cells were obtained at days E 14.5 and E 15.5. They were LacZ (β-galactosidase) stained "en bloc" and cross-sectioned head to tail. In esophagus, trachea, lung, stomach, hindgut, and the future urinary bladder, we observed various mesoderm-derived β1-negative cells (e.g. fibroblasts, chondrocytes, endothelial cells, and smooth muscle cells) but no β1-negative epithelial cells. Since the epithelia of lung, esophagus, trachea, stomach, hindgut, and urinary bladder are derived from the endodermal gut tube, we hypothesize that β1 is essential for the development and/or survival of the epithelia of the fore- and hindgut and its derivatives.

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Introduction

The primitive gut tube is formed during cephalocaudal and lateral folding of the embryo (in mice embryonic days E 8–E9). The lung forms subsequently through outpouching of the foregut endoderm into the splanchnic mesoderm. In the mouse, the embryonic period of lung development occurs between E 9.5 and E 12 and results in the formation of the major future airways. Esophagus and trachea form by a division of the foregut at E 10 to E 11 (Burri 1999; Kaufman 1995). The development of the stomach starts with the appearance of the gastric dilatation in the abdominal foregut (E 10–E 10.5), followed by rotation and curvature of the stomach (Kaufman 1995). At day E 12 to E12.5 the cloacal region is starting to become divided by the downward growth (towards the cloacal membrane) of the urorectal septum, resulting in the formation of the urogential sinus anteriorly (future urinary bladder) and the hindgut posteriorly (Kaufman 1995).

Following the embryonic period of lung development branching morphogenesis continues at the tips of the bronchial tree during the pseudoglandular stage (E 12–E 16.5). At the end of the canalicular stage (E 16.5–E 17.5) branching of the airways is completed and the epithelial cells have differentiated into type I and II alveolar cells. The saccular stage (E 17.5–P 4) results in expansion of the air spaces, and after birth, alveolarization (P 4–P 14) and microvascular maturation (P 14–P 21) take place (Ten Have-Opbroek 1991; Schittny et al. 2003).

Integrins are a large family of transmembrane cell-surface receptors (Hynes 1992), composed of a single α and β peptide subunit (Hynes 1992). Integrins are able to bind extracellular matrix proteins, such as collagens, fibronectin, and laminins, and cellular receptors, such as vascular cell adhesion molecule-1 and the intercellular adhesion molecules family (Plow et al. 2000). The β1

integrin (β1) associates with at least 12 different α subunits and thus forms the largest subfamily of integrins (Bouvard et al. 2001). In humans, the β1 can be expressed in at least four variants, termed β1A, β1B, β1C, and β1D, which differ in their cytoplasmic domains (Sheppard 2000). Members of the β1 family are expressed almost on all cells, however, their composition is cell type specific. This specificity is believed to play an important role in the developing embryo. The extracellular domain of the α subunit confers the binding specificity of β1, while the cytoplasmic domains are responsible for a particular biological response to binding (Chan et al. 1992; Kassner et al. 1993; Kawaguchi et al. 1993). The cytoplasmic domains interact with downstream signal transduction molecules, such as focal-adhesion kinase and integrin-linked kinase (Hannigan et al. 1996; Schaller et al. 1995), and with the cytoskeleton by associating with actin filament-binding molecules (Chen et al. 1995; Otey et al. 1993). These interactions can affect shape, migration, differentiation, and proliferation of cells.

Homozygous β1-deficient embryos develop normally to the blastocyst stage, implant and invade the uterine basement membrane but die shortly afterwards. It has been suggested that fertilization and preimplantation of β1-deficient embryos is normal because of the presence of maternal β1 RNA and protein (Brakebusch et al. 1997). Heterozygous β1-deficient mice develop normally and are indistinguishable from wild-type littermates (Fässler and Meyer 1995).

To circumvent the early embryonic lethality of the homozygous β1-deficient fetuses, chimeric mice have been used to study β1 function during development. These mice contained transgenic cells with the *LacZ* gene (β-galactosidase) inserted into the *β1-integrin* gene. As a result, a number of cells expressed LacZ instead of β1 under the control of the β1-integrin promoter. Chimeric mice which contain less than 25% of β1-deficient cells develop normally. No anatomical or morphological alterations were observed either in fetal or in adult mice. Above the 20 to 25% threshold, embryos are distorted (Fässler and Meyer 1995).

In the present study, using LacZ staining of β1-deficient cells, we examined endoderm-derived organs of chimeric mice to explore which cells are dependent on β1 during development.

Materials and Methods

Preparation of chimeric β1-deficient mice

Chimeric mice were obtained according to Fässler and Meyer (1995). Briefly, embryonic stem (ES) cell lines D3 and R1 were used for generating a β1-integrin-null ES cell clone (G201). The insertion of a β-galactosidase-neomycin (geo) fusion DNA in frame with the ATG of the β1-integrin leads to the inactivation of the *β1* gene. Blastocysts from C57BL/6 wild-type mice were isolated at day 3.5 p.c., injected with 15 G201 β1-deficient ES cells (Fässler et al. 1996) as described by Bradley (2001), and transferred into the uterus of pseudopregnant recipient (C57BL/6xDBA) F1 wild-type females (2.5 days p.c.). Embryos were isolated at days E 14.5 and E 15.5, fixed for 30 min in PBS, containing 4% paraformaldehyde and 0.002% NaN_3, washed three times in PBS, and stained "en bloc" overnight at 37ºC in a solution of 1 mg/ml X-gal (Sigma, St. Louis, MO, USA) for LacZ activity following established protocols (Fässler and Meyer 1995; Friedrich et al. 1991).

Handling of the animals before and during the experiments, as well as the experiments themselves, were approved and supervised by the Animal Ethics Committee of the University of Munich (Germany) and by the Swiss Agency for the Environment, Forests and Landscape and the Veterinary Service of the Canton of Bern (Switzerland).

Distribution of LacZ-positive cells

Complete embryos were embedded in paraffin (Histosec, Merck, Darmstadt, Germany) at 60ºC after using graded series of ethanol and Histoclear (Life Science, Frankfurt, Germany) as intermedium. 5 µm serial cross-sections of the whole embryos were obtained head to tail and analyzed for LacZ staining without any further counterstaining.

Immunostaining for β1

For immunoperoxidase staining sections were dewaxed in three changes of Histoclear and a graded series of ethanol, and one change of TBS, fixed by two changes of methanol, containing 1% H_2O_2, treated with trypsin (Sigma) diluted 1:250 in TBS, containing 100 mM $CaCl_2$ at 37°C, and cooked in 0.01 M Na-citrate, pH 6.0, in a microwave oven for 15 min. In the next step, sections were blocked for 30 min with TBS, containing 5% bovine serum albumin and 1% normal goat serum, and incubated with anti-β1-integrin (9EG7, cell culture supernatant) (Lenter et al. 1993) and biotinylated anti-rat IgG (Jackson ImmunoResearch, West Grove, PA, USA), diluted 1:500. After each incubation step, sections were washed three times with TBS. Finally, sections were mounted in Mowiol (PBS, containing 15% of the substance Hoechst 4.88 (Hoechst, Frankfurt, Germany) and 50 mg/ml 1.4-diazabicyclo(2.2.2)octane (Merck, Darmstadt, Germany). Non-specific mouse IgG was used as negative control for β1 staining. Wild-type fetuses were used as negative control for LacZ. In order to show that the LacZ-positive cells did not express any β1 a double staining—immunoperoxidase for β1 and galactosidase for LacZ—was performed. Except for the midgut-derived intestinal epithelium, no double staining for LacZ and β1 was observed (data not shown). The expression of a genuine galactosidase in the midgut-derived intestinal epithelium was too strong to be completely quenched, and therefore, this tissue could not be studied.

For immunofluorescence staining 5 μm mid-sagittal sections of 14.5-day-old embryonic mice were used. Sections were fixed in methanol at −20°C for 5 min. Non-specific protein binding was saturated by incubation with PBS containing 1% bovine serum albumin. Anti β1-integrin (see above) was applied for 60 min. Bound antibodies were visualized using goat anti-rat IgG conjugated with rhodamine (Dianova, Hamburg, Germany). Washing and mounting was done as described above. The same negative controls resulted in no non-specific background activity.

Results

β1 in wild-type animals

In the investigated organs of wild-type mice (esophagus, trachea, lung, stomach, hindgut, and the future urinary bladder), both endoderm-derived epithelial cells and mesoderm-derived mesenchymal cells were β1-positive [own data and (Hemler 1999)]. As an example for these

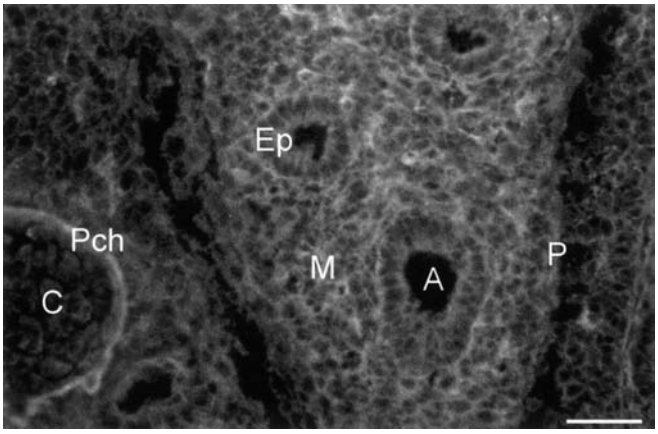


Fig. 1 Immunofluorescence staining of β1 in fetal lung of a wild-type animal on day E 14.5. The lung is in the pseudoglandular stage of development, and both endoderm-derived epithelial cells and mesoderm-derived mesenchymal cells are β1-positive. *A* Airway, *C* cartilage, *Ep* epithelium, *M* mesenchyme, *P* pleura, *PCh* perichondrium. Bar, 50 μm

tissues an immunofluorescence staining of β1 in lung at embryonic day E 14.5 is shown in figure 1. In the lung epithelial cells, intense staining was seen at the cell base towards the basal membrane and at the apex of the cell. In lung mesenchymal cells, no particular pattern of staining could be detected (Fig. 1).

β1 in chimeric animals: whole mount embryos

At embryonic days 14.5 and 15.5 a total of 22 chimeric β1-deficient embryos were LacZ-stained "en bloc", completely sectioned and carefully examined. Before sectioning, the intensity of blue staining of whole mount stained embryos was assessed semi-quantitatively (Table 1). The morphology of each embryo was examined and found to be normal with the exception of one embryo. In this embryo (E 15.5) staining was very strong and associated with a markedly distorted anatomy suggesting a transgenic β1-negative cell content in excess of 25% (Fässler and Meyer 1995).

β1 in chimeric animals: tissue sections

In chimeric β1-deficient mice, LacZ staining revealed clusters of mesoderm-derived β1-negative cells (e.g. fibroblasts, chondrocytes, endothelial cells, and smooth muscle cells) in trachea (Fig. 2a), esophagus (Fig. 2b), lung (Fig. 2c,d), stomach (Fig. 2e), hindgut (Fig. 2f), and the urogenital sinus (future urinary bladder, Fig. 2g). In contrast, we did not observe any β1-negative epithelial cells in any of these tissues (Fig. 2a–g).

In lung, we observed various β1-negative mesenchymal cells. However, parts of the smooth muscle cell layers of large and small airways, as well as the smooth muscle cell layers of blood vessels contained a particularly high number of β1-negative cells. At E 14.5 and E 15.5, the lung of mice is in the pseudoglandular stage of lung development, and the future airways are lined by cuboidal epithelial cells which have not yet differentiated into type I and II cells. None of these endoderm-derived epithelial cells were β1-negative (Fig. 2c,d).

In the trachea (Fig. 2a), esophagus (Fig. 2b), stomach (Fig. 2g), and hindgut (Fig. 2f) scattered mesenchymal cells of the submucosal layer were β1-negative, however, the most intense staining was again observed within their smooth muscle cell layers. No β1-negative cells were found in the mucosal epithelial cell layers of these tissues.

Finally, no cells of the transitional epithelium of the future urinary bladder were β1-negative. Note, at E 15.5, the trigone of the urinary bladder which consists of mesoderm-derived epithelial cells has not yet formed. In all other parts of the urinary bladder LacZ-positive cells were observed (Fig. 2g).

The extent of LacZ staining varied greatly between sections with some showing large areas of β1-negative cells (Fig. 2c–e) and others showing none (Fig. 2b). This uneven distribution of β1-negative cells suggests a clonal origin of these cells and is particularly striking for smooth muscle cells (Fig. 2c–e). A similar clonal appearance of β1-integrin negative cells was observed in exclusively mesenchymally-derived tissues. For example, in cartilage, LacZ staining was evident in the perichondrium and on single as well as isogenic groups of chondrocytes (Fig. 2h). Comparing days E 14.5 and E 15.5 no principle difference was observed in terms of the appearance and distribution of β1-negative cells.

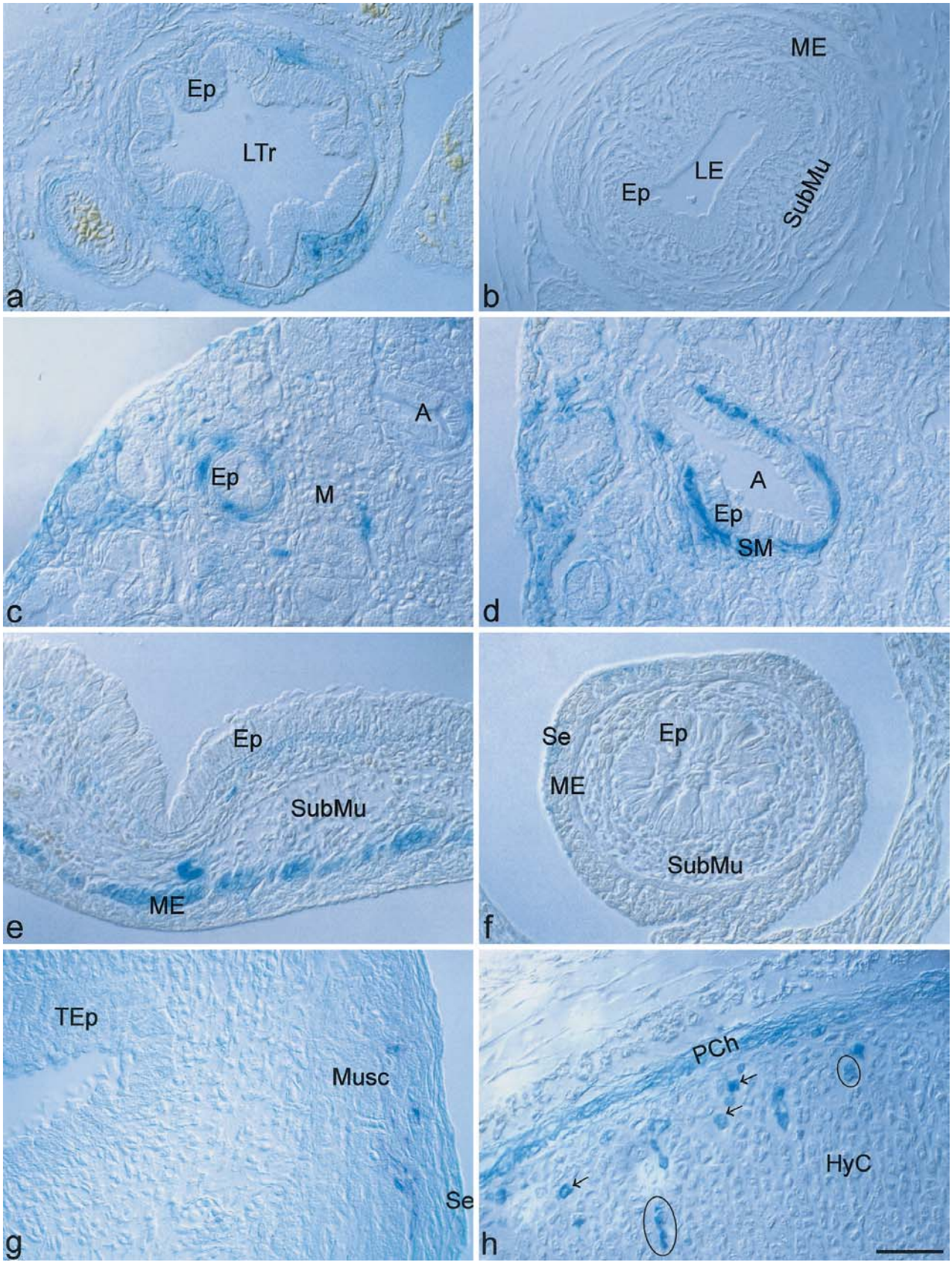
Discussion

As shown for lung in the present paper (Fig. 1), almost all cells of the body are β1-integrin (β1) positive in wild-type mice (Hemler, 1999). β1 appears to be essential for embryonic development, because homozygous β1-null embryos die shortly after implantation. In vitro, β1-negative embryonic stem (ES) cells show altered adhesion and an impaired ability to migrate on extracellular matrix

Table 1 Grading of LacZ staining

LacZ staining	None	Moderate	Good	Strong	Very strong	Subtotal	Total
E 14.5	0	1	1	5	0	7	22
E 15.5	2	3	5	4	1[a]	15	

The intensity of blue staining was assessed semi-quantitatively on whole mount stained embryos. In addition, the morphology of each embryo was evaluated. No alterations were observed except in one embryo which was stained very strongly ([a]). The two unstained embryos were used as negative controls


Ep
LTr
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HyC
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proteins (Fässler et al. 1995). These cells lose their tight cell–cell contacts, become round and acquire microvilli-like structures. The cytoskeleton, however, appears to be unaffected by these changes. Surprisingly, ES cells can integrate themselves into the inner cell mass (ICM) of blastocysts and contribute to most ectodermal and mesodermal lineages. In early embryos they were also found in the endoderm (Fässler et al. 1995; Fässler and Meyer 1995). The resulting β1-negative chimeric mice developed normally as long as they did not contain more than ~25% transgenic cells. The latter cells were able to migrate and differentiate in contact to wild-type cells. Later, high levels of β1-negative cells were found in skeletal muscle and gut, low levels in lung, heart, and kidney, but none in liver and spleen (Fässler and Meyer 1995).

In our present study we investigated which cells of endodermally-derived organs tolerate β1-negative cells in their lineage. At embryonic days E 14.5 and E 15.5 we obtained chimeric β1-null mice according to Fässler and Meyer as described above (Fässler and Meyer 1995). We utilized LacZ-staining (galactosidase) to identify β1-deficient cells in serial head to tail cross-sections of the embryos.

We observed β1-negative cells in the connective tissues, blood vessels, and the smooth muscle layers in all of the investigated endoderm-derived organs (trachea, esophagus, lung, stomach, hindgut, and urinary bladder). They appeared in a scattered or clustered pattern. This was particularly striking for smooth muscle cells (-precursor) and fibroblasts (Fig. 2a–g). In contrast, no β1-negative cells were detected in the endoderm-derived epithelia of trachea, esophagus, lung, stomach, hindgut, and urinary bladder (Fig. 2a–g).

Our results indicate that mesoderm-derived β1-negative cells of the investigated organs are able to migrate, differentiate and survive as long as a significant surplus of wild-type cells is available. Apparently, in those tissues the wild-type cells are able to organize the required pattern of growth factors and the architecture of the extracellular matrix as long as less than ~25% of β1-negative cells are present (Fässler and Meyer 1995; Bagutti et al. 2001). β1-defiency affects cellular migration and adhesion (Fässler et al. 1995). Most likely a β1-dependent collective cell movement (movement of cells in clusters) is not possible anymore. But β1-null cells may switch to a β1-independent "amoeboid" crawling and circumvent this defect (Hegerfeldt et al. 2002). In addition, other integrins may be able to compensate for the absence of β1 and contribute to cellular movements.

Similar behavior of β1-negative cells was observed for skeletal muscle cells. Even if antibody perturbation experiments have suggested an important role of β1 for migration of muscle precursor cells from the dermomyotome to the limbs (Jaffredo et al. 1998), β1-negative myoblasts migrate, form myotubes, and develop a normal sarcomeric cytoarchitecture as long as there are not more than 25% of β1-negative cells present in chimeric mice (Hirsch et al. 1998). In contrast, in skeletal muscle specific β1 knockouts (all myoblasts are β1-negative) myoblast fusion is defective. Therefore, the dominant fraction of wild-type myoblasts is able to rescue the β1-defency in this chimeric mouse (Schwander et al. 2003).

In contrast to ectoderm- and mesoderm-derived cells, β1 appears to be essential for the development and/or survival of endoderm-derived epithelial cells (Fässler and Meyer 1995). Different explanations may be given, but all of them remain speculative. (1) It may be that for the development of these epithelia a β1-dependent collective cellular movement is required. (2) The interplay between integrins and growth factors is altered during the differentiation/development of β1-null keratinocytes. Apparently, higher levels of growth factors are required by these keratinocytes (Bagutti et al. 2001). A similar phenomenon may prevent endodermal β1-deficient cells to differentiate into epithelial cells of the primitive gut and its derivatives. (3) The recognition of extracellular matrix components is needed for the proper differentiation and especially for the maintenance of cardiac muscle cells in β1-null chimeric mice. In chimeric β1-null cardiac muscle Fässler et al. (1996) observed that the number of β1 -/- muscle cells decreased during postnatal life. They observed cellular debris, but no real apoptosis. Similarly, it may be possible that β1-deficient cells are not maintained after an early integration into the epithelium of the primitive gut tube.

Perl et al. (1999) showed that even before the appearance of the lung buds a lineage restriction in the endoderm takes place. For example, for most of the peripheral lung epithelial cells, determination already takes place at days E 4.5 to E 6.5 and for trachea and bronchi at days E 9 to E 9.5. As β1-deficient cells are still present in the endoderm during lineage restriction (Fässler and Meyer 1995), we speculate that at least a certain amount of β1-null cells is tolerated during this process.

In summary, our observation suggests that: (1) for the development and/or survival of epithelial cells of endoderm-derived organs β1 is essential and that (2) rescue mechanisms which allow β1-negative cells to participate in the development of the mesoderm-derived part of these

Fig. 2a–h Chimeric animals were LacZ-stained at E 15.5. In organs containing endodermally derived epithelia LacZ staining of β1-negative cells was observed in trachea (**a**), esophagus (**b**), lung (**c,d**), stomach (**e**), hindgut (**f**), and the urogenital sinus (future urinary bladder; **g**). In all of these organs β1-negative cells were present in the mesoderm-derived cells, but not in the endoderm-derived epithelia. The concentration of β1-negative cells varied greatly between different sections. In some sections, a large extent was detected (**c,d**) and in other sections none were visible (**b**), suggesting a clonal origin of these cells. This was particularly evident in the case of smooth muscle cells (**c–e**). The clonal origin was also evident in the isogenic groups of chondrocytes (chondron) in hyaline cartilage (**h**). *A* Airway, *Ep* epithelium, *LE* lumen of esophagus, *LTr* lumen of trachea, *M* mesenchyme, *ME* muscularis externa, *Musc* muscularis, *HyC* hyaline cartilage, *PCh* perichondrium, *Se* serosa, *SM* smooth muscle, *SubMu* submucosa, *TEp* transitional epithelium; a*rrow* single chondrocyte, *circle* chondron. Bar, 50 μm

organs are insufficient for the endoderm-derived epithelia.

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